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PURIFICATION AND PROPERTIES OF MOUSE SERUM CARBOXYLIC ESTER HYDROLASE (EC 3.1.1.1) (U)

by

J.G. Clement

PCN No. 351SD

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ABSTRACT

The purification procedure and properties of mouse serum carboxylic ester hydrolase (EC 3.1.1.1) are reported. The enzyme was purified using hydrophobic interaction chromatography followed by anion exchange fractionation and chromatofocusing. The purity of the enzyme was assessed by polyacrylamide gel electrophoresis and isoelectric focusing. The effect of various ions (Na, Ca, Mg, Mn, Cu, K, Zn, Li, Ce, Fe), organophosphate (DFP, soman, sarin, tabun and VX) and carbamate (physostigmine) anticholinesterases and CBDP (2-/O-cresyl/-4H-1:3:2-benzodioxaphosphorin-2-oxide)

ABSTRACT (Cont'd)

on the carboxylic ester hydrolase activity were examined. Generally the organophosphate anticholinesterases, with the exception of VX, were potent inhibitors of carboxylic ester hydrolase activity whereas the carbamate, physostigmine, was a poor inhibitor. The various ions inhibited carboxylic ester hydrolase activity. None of the ions stimulated carboxylic ester hydrolase activity. Further research is in progress investigating the interaction of nerve agents with carboxylic ester hydrolase and developing purification schemes for carboxylic ester hydrolase from other sources.

ACKNOWLEDGEMENTS

The author would like to acknowledge the assistance provided by Dr. George Drummond (Deceased, University of Calgary), who introduced the author to the joys and frustrations of column chromatography and protein purification. In addition, Mr. A. Hansen synthesized various chemicals used in this study.

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INTRODUCTION

It was previously reported from this laboratory that plasma carboxylic ester hydrolase (E.C. 3.1.1.1; aliesterase) was an extremely important *in vivo* detoxification route for soman in rodents (Clement, J.G., 1984a, Clement, J.G., 1983). If the level of serum carboxylic ester hydrolase was reduced, the animal became more sensitive to the toxic effects of nerve agent exposure (*loc. cit.*). In contrast, if the serum levels of carboxylic ester hydrolase were increased, by inducing liver hyperplasia through phenobarbital pretreatment, a protective effect against nerve agent exposure was obtained (Clement, J.G., 1984b). The purpose of this investigation was to develop a purification scheme for mouse serum carboxylic ester hydrolase and to investigate the basic biochemical properties of the enzyme and its interaction with various inhibitors.

MATERIALS

Trace hemolysed frozen mouse serum was obtained from Pel-Freeze Biologicals (Rogers, Arkansas). The serum was obtained from Swiss-Webster mice. Physostigmine sulfate, Bis-Tris, tributyrin, p-nitrophenyl acetate (PNPA), p-nitrophenyl butyrate (PNPB), phenyl methylsulfonylfluoride (PMSF), β-naphthylacetate (β-NPA), α-naphthylacetate (α-NPA) and Fast Blue BB salt were obtained from Sigma Chemical Co. Diisopropylfluorophosphate (DFP) and butyrylthiocholine were obtained from Aldrich Chemical Co.

The Organic Chemistry Group at Defence Research Establishment Suffield synthesized soman (pinacolyl methylphosphonofluoridate), sarin (isopropyl methylphosphonofluoridate), tabun (dimethyl phosphonoamido cyanidate), CBDP (2-/O-cresyl/-4H-1:3:2-benzodioxaphosphorin-2-oxide), VX (((O-ethyl-S-(2-diisopropylamino)ethyl)methylphosphonothioate)) and ^{14}C-methyl butyrate (SA = 64 mCi/mole).

All other common chemicals were reagent grade and obtained from commercial suppliers.

ENZYME ASSAYS

a) PNPA Assay:

Reactions were carried out in a final volume of 2.02 mL. Tris buffer (1.0 mL; 20 mM; pH 8.0) and enzyme (20 μL) was added followed by substrate solution (1.0 mL) of PNPA (0.88 mM, final concentration). Enzyme activity was monitored by change in absorbance at 405 nm and 25°C. The substrate solution was prepared by dissolving PNPA in 2.0 mL of acetonitrile and adding this slowly with mixing to distilled water. The presence of acetonitrile did not affect enzyme activity.

b) Cholinesterase Assay:

The procedure employed was based on that of Ellman *et al.*(1961) using butyrylthiocholine (4 mM) in sodium phosphate buffer (100 mM; pH 6.8) as the substrate and measuring the change in absorbance at 405 nm and 30°C.

c) Tributyrin Assay:

Tributyrin hydrolysis was assessed by the pH-stat technique. To a reaction vessel (23°C), tributyrin (10 mL of 0.2% solution which was well mixed) was added and the solution was stirred vigorously and titrated to pH 7.9 using a Titragraph® (Radiometer, Copenhagen). To start the reaction, 50 μL of enzyme solution was added. Rate of addition of 0.01 N NaOH from the first to the fifth minute was used in determining enzyme activity. The reaction was linear over this time period. Solutions were degassed by bubbling pure nitrogen through them for at least 5 min and the reaction vessel was purged with nitrogen while the reaction was in progress.

d) PNPB Assay:

The general procedure of Sterri *et al.*, 1985 was used. To 1.0 mL of sodium phosphate buffer (100 mM; pH 7.8) 10 μL of enzyme was added followed by 1.0 mL of the substrate PNPB (2.0 mM). Enzyme activity was monitored by change in absorbance at 405 nm and 30°C. During the assay the substrate solution was stirred continuously to ensure a homogeneous solution. The substrate solution was prepared by dissolving the PNPB in acetonitrile and adding this slowly to distilled water while mixing continuously.

e) ^{14}C-methyl butyrate Assay:

The procedure used was essentially that of Sterri *et al.*, 1985. Briefly, ^{14}C-methyl butyrate solution (1.9 mM) was incubated with enzyme (25 μL) in 0.5 mL of phosphate buffer (100 mM; pH 7.8) for 20 min at 30°C. The reaction was terminated by addition of 0.5 mL of ice-cold phosphate buffer and 1.0 mL of chloroform. The mixture was vortexed, centrifuged (to separate phases) and 0.5 mL of the aqueous phase was added to 10 mL of scintillation fluid and counted on a Beckman 9800 liquid scintillation counter. The unhydrolysed substrate was extracted into the chloroform phase and the butyric acid anion remained in the aqueous phase to be counted. The reaction was performed in glass stoppered 2 mL volumetric tubes.

f) β-NPA Assay:

The procedure was based on that of Burlina *et al.*, 1977. Briefly, to 1.0 mL of β-NPA solution (0.27 mM) in a curvette was added 2.5 μL of enzyme solution which was mixed thoroughly and the change in absorbance was monitored at 328 nm and 25° C for 3 min. The rate of change in absorbance between 30 – 90 sec was used in calculating reaction rate. The substrate solution was prepared by dissolving β-NPA in dimethyl-formamide and adding this slowly to Tris buffer (50 mM; pH 7.2) with vigorous stirring.

g) "Somanase" Activity:

Phosphorylphosphatase activity results in the release of fluoride from soman. Hydrolysis of soman by phosphorylphosphatase action was monitored using a fluoride sensitive electrode similar to the procedure of Hoskin and Roush (1982).

Soman (3 mM) was dissolved in solution of 400 mM KCl, 50 mM NaCl and 5 mM Bis-Tris, pH 7.2. The enzyme was added and solution stirred continuously while the rate of increase in fluoride concentration was recorded.

ENZYME PURIFICATION

Column chromatography was carried out at room temperature using a Pharmacia FPLC chromatography system. Protein concentration was determined according to the method of Bradford (1976) using reagents supplied by Bio-Rad (Richmond, Ca).

a) Hydrophobic Interaction Chromatography:

The 40% saturated ammonium sulfate (SAS) supernatant of pure serum was applied directly to a column (2.6 × 38 cm) containing phenyl-sepharose CL-4B (Pharmacia) which was equilibarated with 20 mM sodium phosphate buffer in 4 M NaCl (pH 8.0). The column was then washed with 255 mL of this buffer. A major, broad protein peak was eluted from the column by reducing the ionic

strength linearly over the next 195 mL to sodium phosphate buffer (20 mM; pH 8.0) and washed with an additional 310 mL of the latter. The major enzyme activity was eluted using a 20 mM sodium phosphate buffer in 50% ethylene glycol (pH 8.0).

b) Anion Exchange Collumn:

The buffer of the major enzyme peak from the phenyl-sepharose column was exchanged using a Pharmacia PD-10 column to Tris buffer (50 mM, pH 8.0) and applied to a anion exchange column (Mono Q HR 10/10; 1.0 × 10 cm, Pharmacia) at a flow rate of 2.0 mL/min and washed on with a total volume of 50 mL Tris buffer. The enzyme was then eluted using an increasing NaCl gradient (Tris buffer, 50 mM, in 500 mM in NaCl, pH 8.0) over the next 120 mL.

c) Chromatofocusing:

Chromatofocusing is a unique column chromatographic method for separating proteins according to their isoelectric points. The buffer of the combined enzyme fractions from the anion exchange step was exchanged into Bis-Tris buffer (25 mM, pH 7.4) and loaded onto a chromatofocusing collumn (Mono P HR 5/20 containing Polybuffer Exchange 94; 0.5 × 20 cm; Pharmacia). The column was then eluted with Polybuffer 74® (diluted from stock 1 : 10 and pH adjusted to 4.0 with iminodiacetic acid; Pharmacia) to form the pH gradient. Following development of pH gradient to pH 4.0, the column was then washed with sodium phosphate buffer (20 mM in 4.0 mM NaCl, pH 8.0) to elute the enzyme.

ELECTROPHORESIS

Electrophoretic purity of the enzyme was assessed using polyacrylamide gradient gel electrophoresis (Pharmacia gradient gels; PAA 4/30). Five mL of a sample of diluted (1 : 10) pure serum and a sample of diluted (1 : 10) semipurified carboxylic ester hydrolase from the chromatofocusing step was applied to the gel along with a high molecular weight protein calibration standard (Pharmacia) in a Tris (90 mM)-borate (80 mM)-Na_2 EDTA

(3 mM) buffer (pH 8.35). The plates were run in a Pharmacia GE-214 gel electrophoresis apparatus with temperature maintained at 14° C. Run in conditions were 20 min at 70 V with constant voltage followed by 15 hr at 125 V with constant voltage (1900 volthours) using a Pharmacia Electrophoresis constant Power Supply (ECPS 3000/150) and volthour integrator. The gels were fixed, stained with Coomassie Blue and destained for visualization of protein bands (see below). Some gels were stained for carboxylic ester hydrolase activity using α-NPA (see below).

ELECTROPHORESIS STAINING

a) Protein:

Gels were fixed in a 10% sulphosalicylic acid solution for 30 min. Following fixation, the gels were stained using Coomassie Blue R-250 (4 g/L) in methanol : acetic acid : water (25 : 10 : 65 V/V/V) overnight followed by destaining in methanol : acetic acid : water (25 : 10 : 65 V/V/V).

b) Carboxylic Ester Hydrolase:

The polyacrylamide gel was washed in Tris buffer (50 mM; pH 7.4) for 20 min then stained as follows. α-NPA (75 mg) was dissolved in 1 mL of dimethylformamide (DMF) and added directly with mixing to 100 mL of Tris buffer in the staining dish. To this was added 100 mg of Fast Blue BB salt dissolved in 1 mL of DMF, the solution was shaken using an orbital shaker and the gel added immediately and stained for 15 min. The gel was then transfered to fixing solution of sulphosalicylic acid (10%) for 30 min then to the methanol : acetic acid : water destaining solution until a clear background was obtained.

ISOELECTRIC FOCUSING (IEF)

IEF of purified carboxylic ester hydrolase from the chromatofocusing step was performed using a Pharmacia (FBA 3000) flat bed IEF apparatus. Twenty μL of carboxylic ester hydrolase was applied to a commercially prepared Resolve® (Iso Lab Inc., Akron, Ohio) IEF agarose gel (pH 3 – 10; 100 × 140 × 1 mm). Pharmacia®

IEF calibration standard (pH 3 – 10) was also included. The gel running conditions were at a constant power of 15 watts and a limiting volatage of 1000 volts for 1488 volthours at 4°C. The gel was fixed in a solution of 10% trichloroacetic acid for 10 min, followed by distilled water rinsing for 60 min. The gel was dried, then stained with Coomassie Blue and destained for visualization of protein bands.

RESULTS AND DISCUSSION

The results illustrated in Figure 1 show the fractionation of the 40% SAS supernatant on a phenyl sepharose column. As can be seen the majority of the carboxylic ester hydrolase activity eluted when the column was washed with a high concentration of polyethylene glycol. This indicates that the enzyme is very hydrophobic in nature. Use of a higher concentration of polyethylene glycol (75%) failed to elute further carboxylic ester hydrolase activity.

The combined fractions from the phenyl sepharose column were then applied to a PD-10 column which had been conditioned with Tris buffer (50 mM; pH 8.0). The fractions from this step were then chromatographed on an anion exchange column (Figure 2) and eluted with an increasing NaCl gradient. The combined fractions from the anion exchange column were buffer exchanged to Bis-Tris (25 mM; pH 7.4), applied to a chromatofocusing column (Figure 3) and eluted with Polybuffer 74. As the pH of the eluant decreased, protein which was devoid of carboxylic ester hydrolase activity was eluted from the column. Increasing the ionic strength and pH eluted the major protein band which contained the carboxylic ester hydrolase activity.

The purification table for the various column chromatographic steps showing the recovery and substrate specificity at the various stages is presented in Table I. From the various substrates examined for determination of carboxylic ester hydrolase activity, PNPA appeared to be the easiest to work with and provided adequate representation of the purification process. This purification scheme provided a purification factor of approximately 86 fold with a recovery of 18.5%.

The purification process also removed the phosphorylphosphatase enzyme which hydrolyzes the non-toxic isomers (C+P+ and C−P+) of racemic soman (Figure 4).

Electrophoresis of the sample obtained from the chromatofocusing step revealed only 2 bands which stained for carboxylic ester hydrolase activity using the substrate α-NPA and for protein using Coommasie Blue. Upon comparison of the location of the carboxylic ester hydrolase bands with the high molecular weight standards, the semipurified carboxylic ester hydrolase had a molecular weight of approximately 65,000 daltons. Isoelectric focusing of the chromatofocusing sample indicated that the carboxylic ester hydrolase had a pI which was less than 3.0 which was consistent with the results from the chromatofocusing step where the carboxylic ester hydrolase activity did not elute from the column at a pH of approximately 4.1.

The results in Table II show the effect of various ions on the activity of carboxylic ester hydrolase. None of the ions stimulated the activity of carboxylic ester hydrolase whereas an increasing inhibition was noted as the concentration of the ions was increased from 0.1 to 1.0 mM. The fact that the activity was not stimulated by divalent cations such as Ca^{++} and Mn^{++} suggested that the enzyme activity was not due to arylesterase activity (Ahmad and Forgash, 1976).

The effect of various inhibitors on the activity of carboxylic ester hydrolase was evaluated (Table III). As can be seen, CBDP and the various nerve agents (soman, sarin, tabun and DFP) were potent inhibitors of carboxylic ester hydrolase activity, whereas the carbamate anticholinesterase, physostigmine, and the protease inhibitor, PMSF, were very poor inhibitors. VX was a markedly less potent inhibitor than the other nerve agents. This might have some bearing on the relative toxicities of the various nerve agents *in vivo*. If the nerve agents have a high affinity for carboxylic ester hydrolase as indicated by their K_i value, then they are bound to this enzyme in preference to acetylcholinesterase. Therefore, if more of the nerve agent is bound to carboxylic ester hydrolase, more of the agent has to be administered in order to produce a toxic dose. This is illustrated in the case of VX, which has a low affinity for the carboxylic ester hydrolase, however, *in vivo* is very toxic (LD_{50} 27 μg/kg; sc in mice), whereas, soman, which has a high affinity for carboxylic ester hydrolase, has an LD_{50} value of 130 μg/kg (sc in mice).

Additonal studies (Clement *et al.*, 1987) using the semipurified carboxylic ester hydrolase preparation, have shown that the various isomers of soman react with the enzyme in a similar stereospecific manner as that which occurs with acetylcholinesterase, namely the P(−) isomers are more potent inhibitors than the P(+) isomers.

Future studies will address the purification of the carboxylic ester hydrolase present in primate and/or human liver in order to compare the biochemical properties of the enzymes from these two species to those of the mouse. If the biochemical properties are similar to those of the mouse enzyme, then perhaps this enzyme can perform a nerve agent detoxification role in the primate species and perhaps even humans.

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Table I

CARBOXYLIC ESTER HYDROLASE PURIFICATION TABLE

FRACTION	Total Protein (mg)	PNPA			TRIBUTYRYN			METHYL BUTYRATE			PNPB			β-NPA			CHOLINESTERASE
		Total Activity	SA [1]	PF [2]	Total Activity	SA [3]	PF	Total Activity	SA [4]	PF	Total Activity	SA [1]	PF	Total Activity	SA [1]	PF	Total Activity
Serum	2,797	402,420	144	1.0	36,000	12.9	1.0	12,316	4.4	1.0	491,880	175.9	1.0	1,256,640	449	1.0	121,140
40% SAS	2,617	406,742	155	1.08	35,152	13.4	1.04	12,008	4.6	1.04	462,935	177	1.0	—	—	—	125,258
Phenyl Sepharose	304	194,025	638	4.4	31,305	103	8.0	4,355	14.3	3.3	257,505	847	4.8	682,485	2,245	5	12,045
Mono Q	33.8	119,956	3,553	24.7	11,720	347	26.9	4,131	122.2	27.8	128,974	3,816	21.7	427,181	12,638	28.1	3,840
Chromato-focusing	6.03	74,382	12,335	85.7	9,519	1,587	123	1,095	181.6	41.3	78,546	13,026	74.1	257,823	42,757	95.2	36

1 SA – specific activity = MIU/mg protein/min

2 PF – purification factor

3 nmoles tributyrin hydrolysed/mg protein/min

4 nmoles methyl butyrate hydrolysed/mg protein/min

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Table II

EFFECT OF VARIOUS SALTS ON CARBOXYLIC ESTER HYDROLASE ACTIVITY[1]

Salt	% Control Activity	
	Concentration	
	1 mM	0.1 mM
NaCl	70	88
KCl	66	85
$MgCl_2$	60	82
$MnCl_2$	59	89
ZnCl	51	80
$CaCl_2$	71	86
NH_4Cl	43	79
LiCl	63	83
CeCl	102	75
$FeCl_3$	25	79
$CuS0_4$	36	88
NaF	82	92

1 Reaction was carried out using PNPA (0.88 mM) as the substrate. There was a 1 min preincubation of the salt with the enzyme prior to addition of the substrate. Blanks were run in the absence of enzyme to correct for any effect of the salt on the hydrolysis of PNPA.

Table III

INHIBITION CONSTANTS OF VARIOUS COMPOUNDS ON PURIFIED MOUSE SERUM CARBOXYLIC ESTER HYDROLASE[1]

Compound	K_i
Sarin	6.84 nM
CBDP	12.48 nM
DFP	20.89 nM
Soman	22.39 nM
Tabun	32.03 nM
VX	4.38 μM
PMSF	19.79 μM
Physostigmine	1.47 mM

1 All compounds were preincubated with enzyme for 5 min before addition of substrate. Two concentrations of the substrate, PNPA (0.88 and 0.44 mM), were used. Reaction was carried out at 25°C.

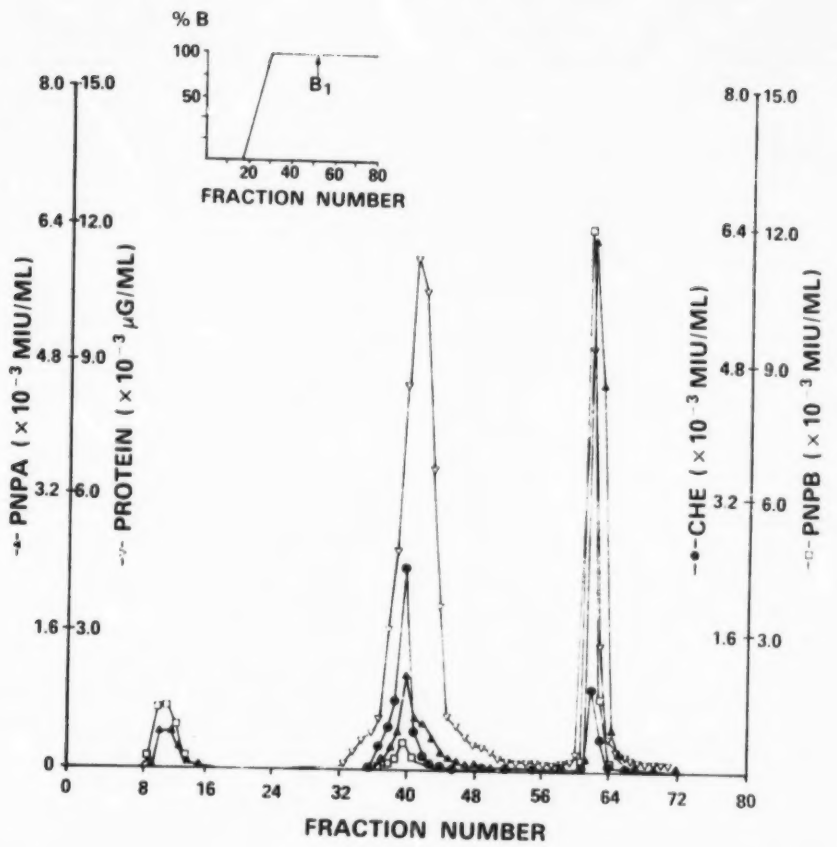

Figure 1

Fractionation of 40% saturated ammonium sulfate supernatant on phenyl-sepharose column (2.6 × 38 cm). Flow rate: 1.0 mL/min. Fraction size = 15 mL. Buffer A = sodium phosphate (20 mM) in 4 M NaCl, pH = 8.0; Buffer B = sodium phosphate (20 mM), pH 8.0; Buffer B_1 = sodium phosphate (20 mM) in 50% ethylene glycol, pH 8.0. Fractions were analysed for PNPA (▲), PNPB (□), cholinesterase (ChE:●) and protein (▽).

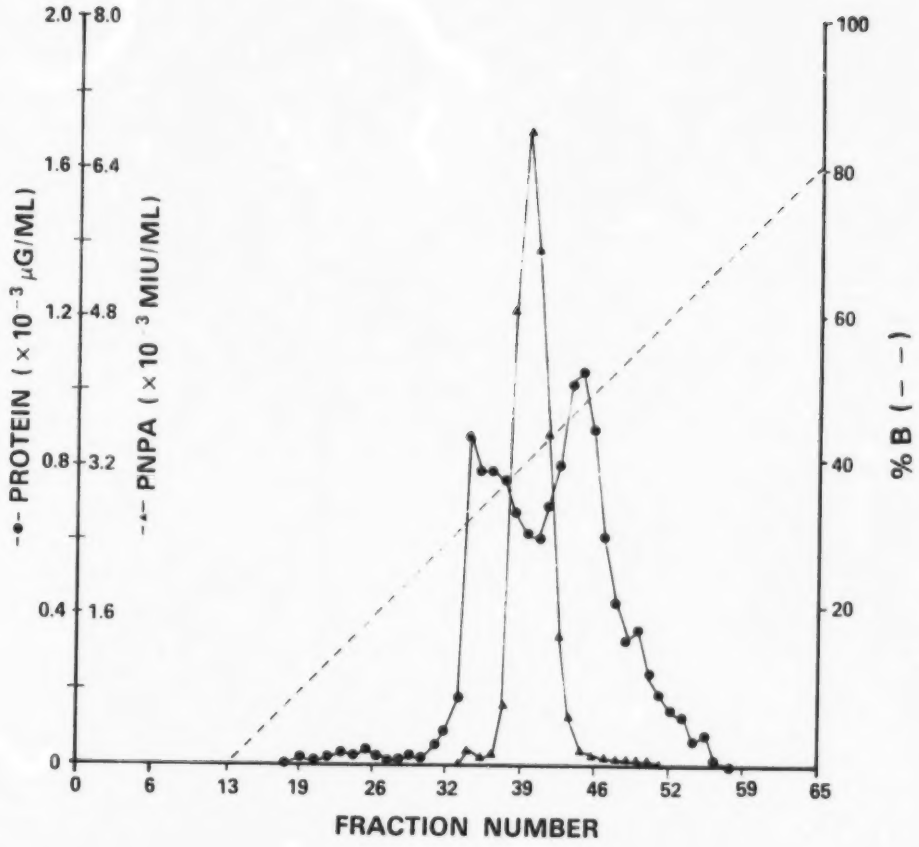

Figure 2

Fractionation of phenyl-sepharose peak carboxylic ester hydrolase activity on anion exchange column (Mono Q HR 10/10). Flow rate = 2.0 mL/min. Fraction size = 4.0 mL. Buffer A = Tris (50 mM), pH 8.0; Buffer B = Tris (50 mM) in 500 mM NaCl, pH 8.0. Fractions were analysed for PNPA and protein.

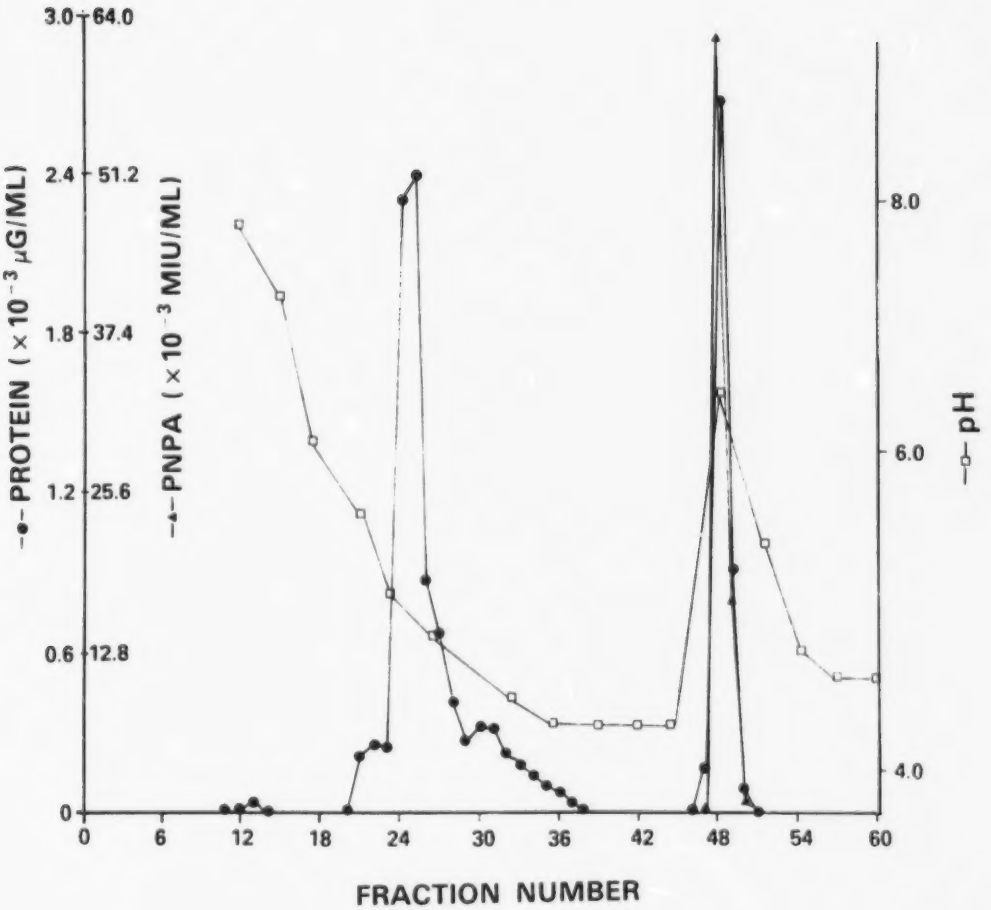

Figure 3

Chromatofocusing of peak column activity anion exchange. (Column, Mono P. HR 5/20) Buffer A, 25 mM Bis-Tris pH adjusted to 7.4 with iminodiacetic acid. Buffer B, Polybuffer 74® diluted 1:10 and pH adjusted to 4.0 with iminodiacetic acid. Buffer B_1, sodium phosphate 20 mM in 4 M NaCl pH 8.0. Flow rate 1.0 mL/min until changed to buffer B_1 (Fraction No. 42) when flow was reduced to 0.5 mL/min. Fractions were analysed for PNPA and protein. The pH was determined using a Pharmacia flow-through pH electrode and monitor.

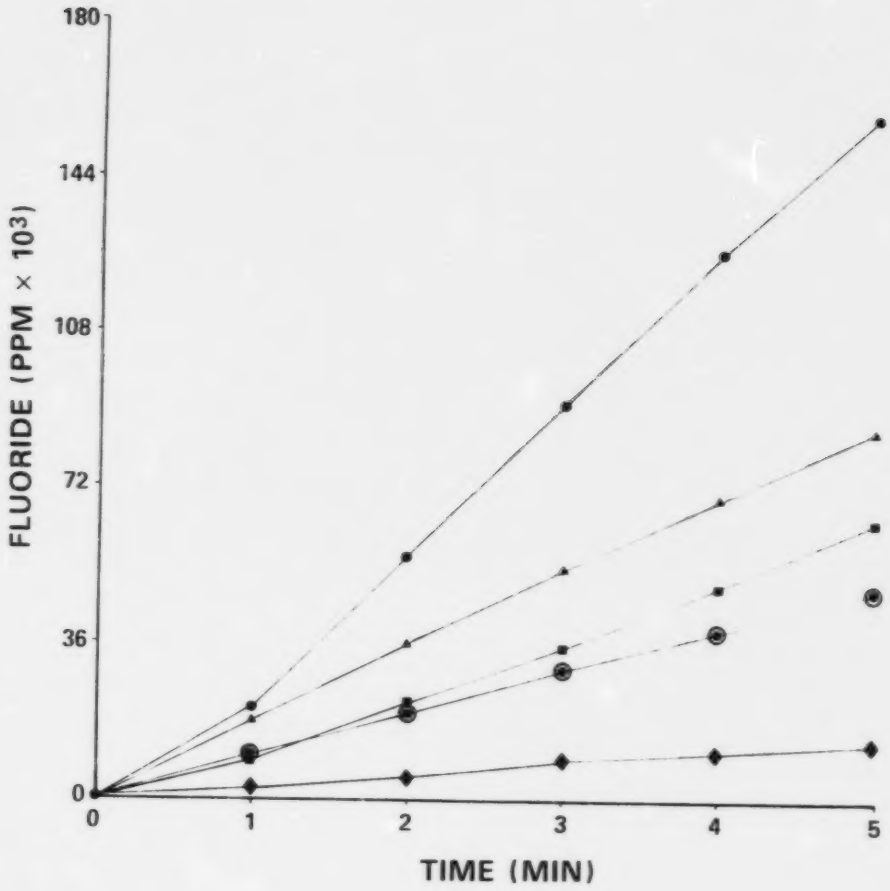

Figure 4

Phosphorylphosphatase hydrolysis of soman by serum and carboxylic ester hydrolase. Pure serum (◆), 10 μL; (■), 25 μL; (▲), 50 μL; (●) 100 μL and carboxylic ester hydrolase 50 or 100 μL (●).

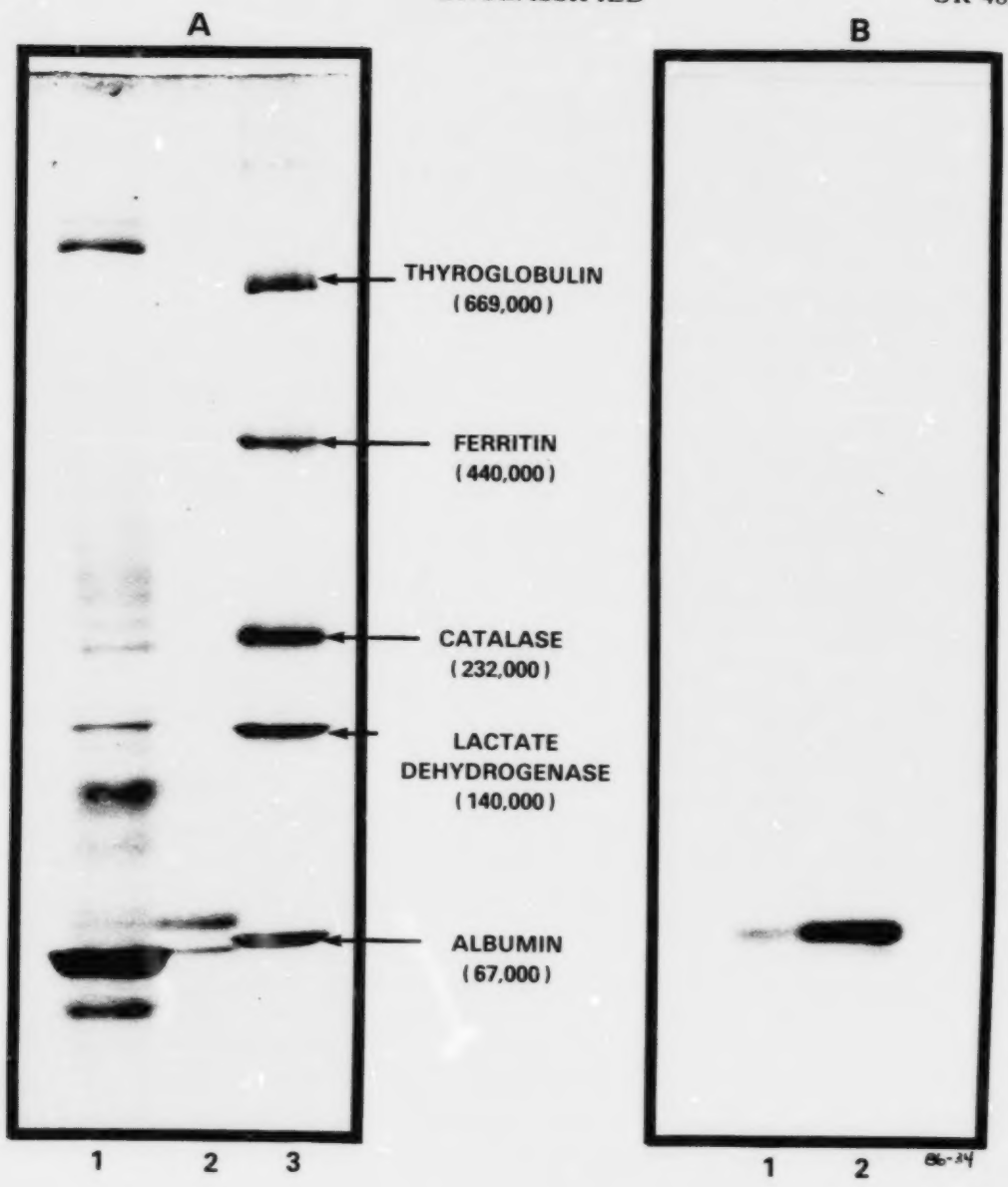

Figure 5

a) **Electrophoresis of mouse serum (Lane 1) and purified carboxylic ester hydrolase (Lane 2) from chromatofocusing sample. Pharmacia high molecular weight calibration standard was present in Lane 3. Protein bands were stained with Coomassie Blue.**

b) **Electrophoresis of pure serum (Lane 1) and purified carboxylic ester hydrolase (Lane 2). Gel was stained for enzyme activity using α-NPA.**

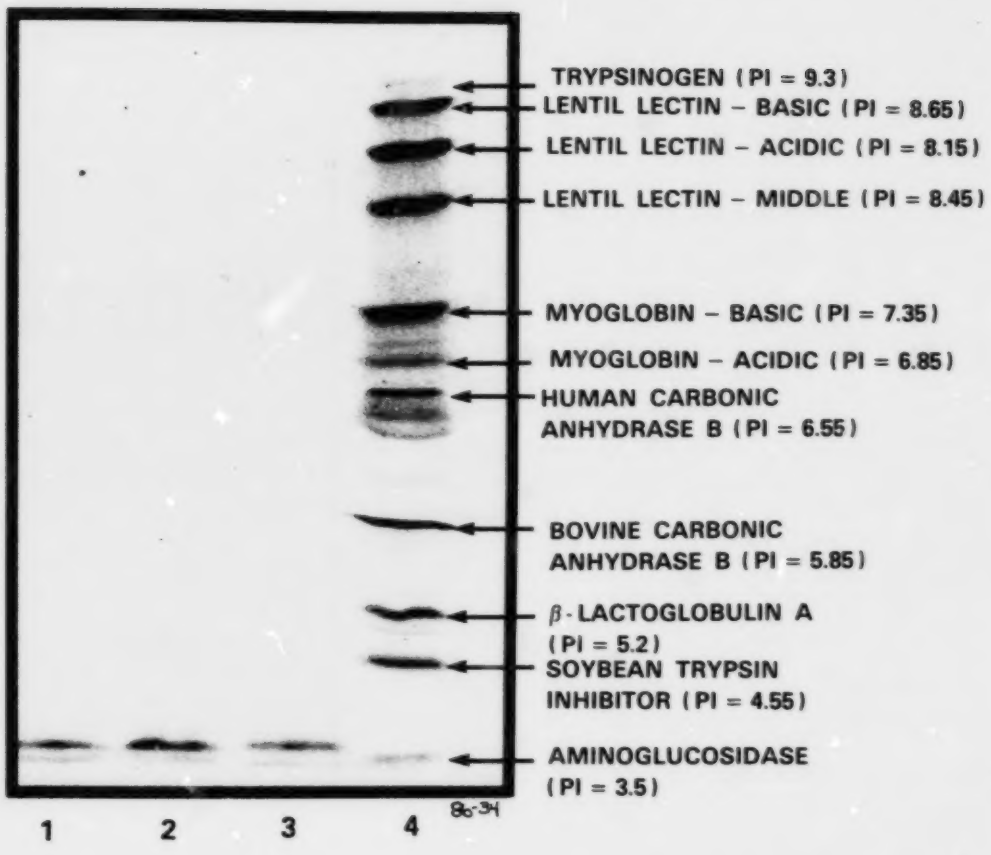
TRYPSINOGEN (PI = 9.3)
LENTIL LECTIN – BASIC (PI = 8.65)
LENTIL LECTIN – ACIDIC (PI = 8.15)
LENTIL LECTIN – MIDDLE (PI = 8.45)
MYOGLOBIN – BASIC (PI = 7.35)
MYOGLOBIN – ACIDIC (PI = 6.85)
HUMAN CARBONIC ANHYDRASE B (PI = 6.55)
BOVINE CARBONIC ANHYDRASE B (PI = 5.85)
β-LACTOGLOBULIN A (PI = 5.2)
SOYBEAN TRYPSIN INHIBITOR (PI = 4.55)
AMINOGLUCOSIDASE (PI = 3.5)
86-34
1
2
3
4

Figure 6

Isoelectric focusing of purified carboxylic ester hydrolase from chromatofocusing sample (Lanes 1, 2, 3) and IEF (pH 3 – 10) calibration standard (Pharmacia) in Lane 4.

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13. ABSTRACT

The purification procedure and properties of mouse serum carboxylic ester hydrolase (EC 3.1.1.1) are reported. The enzyme was purified using hydrophobic interaction chromatography followed by anion exchange gractionation and chromatofocusing. The purity of the enzyme was assessed by polyacrylamide gel electrophoresis and isoelectric focusing. The effect of various ions (Na, Ca, Mg, Mn, Cu, K, Zn, Li, Ce, Fe), organophosphates (DFP, soman, sarin, tabun and VX) and carbamate (physostigmine) anticholinesterases and CBDP (2-/O-cresyl/-4h-1:3:2-benzodioxaphosphorin-2-oxide) on the carboxylic ester hydrolase activty were examined. Generally the organophosphate anticholinesterases, with the exception of VX, were potent inhibitors of carboxylic ester hydrolase activity whereas the carbamate, physostigmine, was a poor inhibitor. The various ions inhibited carboxylic ester hydrolase activity to varying degrees. None of the ions stimulated carboxylic ester hydrolase activity. Further research is in progress investigating the interaction of nerve agents with carboxylic ester hydrolase and developing purification schemes for carboxylic ester hydrolase from other sources.

KEY WORDS

carboxylic ester hydrolase

carboxylesterase

aliesterase

soman

sarin

tabun

VX

nerve agents

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